The Formation of Lithocholic Acid, Chenodeoxycholic Acid and α - and β -Muricholic Acids from Cholesterol Incubated with Rat-Liver Mitochondria

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1. When rat-liver mitochondria were incubated with [4-¹⁴C]cholesterol in the presence of a soluble supernatant fraction, various steroids more polar than cholesterol were formed. These included 3 β -hydroxycholest-5-en-26-oic acid, 3 β -hydroxychol-5-enoic acid, lithocholic acid, chenodeoxycholic acid and α - and β -muricholic acids. 2. All the radioactive C₂₄ bile acids recovered were in conjugated form, probably as taurine conjugates. 3. The formation of 3 β -hydroxychol-5-enoic acid from cholesterol shows that liver mitochondria are capable of carrying out the oxidative removal of the isopropyl unit of the side chain before any modification has occurred in the ring system.

Isolated liver mitochondria catalyse the oxidative cleavage of the side chain of cholesterol (Anfinson & Horning, 1953; Whitehouse, Staple & Gurin, 1959), with the formation of steroids more polar than cholesterol (Fredrickson, 1956; Danielsson, 1960) and of a substance, probably propionyl-CoA, which gives rise to propionic acid on steam-distillation at acid pH (Mitropoulos & Myant, 1965*a,b*). The polar steroids include hydroxylated C₂₄ acids, but none of these has been shown conclusively to be identical with any of the bile acids present in rat bile. In this paper we describe the formation of naturally occurring C₂₄ bile acids from cholesterol incubated with a rat-liver preparation. Since we wished to relate our findings to our earlier observations on the effect of thyroxine on cholesterol metabolism *in vitro* (Mitropoulos & Myant, 1965*a*), we have used a preparation containing liver mitochondria and a soluble supernatant fraction, as in the previous work. A preliminary account of some of this work has already been published (Mitropoulos & Myant, 1966*a*).

METHODS

The rats used were males of the Wistar strain weighing 200–300 g. They were given M.R.C. diet no. 18 (Bruce & Parkes, 1946). The suspensions of liver mitochondria, the soluble supernatant fraction of Whitehouse *et al.* (1959) and the emulsions of [4-¹⁴C]cholesterol were prepared by methods previously described (Mitropoulos & Myant, 1965*a*), except that 1 ml. of the mitochondrial suspension used in the present work contained the mitochondria from 2 g. wet wt. of liver.

The incubation mixture contained: tris-HCl buffer, pH 8.5 (120 mm); ATP (2.7 mm); NAD⁺ (0.5 mm); GSH

(3.2 mm); MgCl₂ (2.6 mm); sodium citrate (4.6 mm); sucrose (10 mm); 1.8 ml. of soluble supernatant fraction; 2.0 ml. of mitochondrial suspension containing 30–40 mg. of mitochondrial protein; 1.0 ml. of [4-¹⁴C]cholesterol emulsion containing 0.1 μ mole; the final volume was 12.0 ml. The incubations were carried out in silicone-treated 50 ml. conical flasks with shaking and with air as the gas phase. At the end of the incubation, 1 ml. of 2*N*-HCl and 20 ml. of ethanol were added to the flasks and the samples were stored overnight at –15°. The contents of each flask were washed into a round-bottomed flask with a further 30 ml. of ethanol and were then evaporated under reduced pressure to a volume of about 2 ml. The acidic aqueous residue was made up to 15 ml. with water and extracted three times with 3 vol. of water-saturated butan-1-ol. The pooled butanol extracts were washed twice with 15 ml. of water.

Kieselgel H was used for thin-layer chromatography. The radioactive materials, dissolved in suitable solvents, were applied to the thin-layer plates with a micro-syringe. After chromatography, radioautographs were prepared by placing the dried plates directly in contact with Ilford Ilfex X-ray film and leaving them in the dark for 1–2 weeks. After radioautography, the plates were stained with the ammonium molybdate-perchloric acid spray of Wagner, Hörhammer & Wolf (1961) to reveal the positions of the markers. Radioactivity was assayed in a Packard Tri-Carb scintillation spectrometer. The bile acid conjugates were hydrolysed by the method of Mosbach, Kalinsky, Halpern & Kendall (1954). The dried eluate from band I (Fig. 1) was hydrolysed by dissolving it in 0.5*N*-KOH in ethanol and heating it on a steam bath under reflux for 3 hr. Methyl esters of free bile acids were prepared by adding an excess of freshly prepared diazomethane in diethyl ether to a solution of the bile acid in diethyl ether-methanol (9:1, v/v). After 5 min. at room temperature the mixture was evaporated to dryness under N₂ at 60°.

Materials. [4-¹⁴C]cholesterol (specific activity 20 μ C/ μ -mole) was obtained from The Radiochemical Centre,

Amersham, Bucks. 7-Oxcholesterol*, cholic acid, hyocholic acid, deoxycholic acid, chenodeoxycholic acid, 3 α ,6 α -dihydroxy-5 β -cholanoic acid, lithocholic acid and 3 β -hydroxychole-5-enoic acid were obtained from Steraloids Ltd., Croydon, Surrey.

3 α ,6 β -Dihydroxy-5 β -cholanoic acid was prepared by the method of Thomas *et al.* (1965).

For the preparation of β -muricholic acid, 3 α ,6 α -dihydroxy-5 β -cholanoic acid was converted into methyl 3 α -acetoxy-6 β -hydroxy-5 β -cholanoate by the method of Thomas *et al.* (1965). This acetoxy compound was dehydrated to methyl 3 α -acetoxy-5 β -chol-6-enoate with POCl₃ in the presence of pyridine (Schubert & Damker, 1957). The product of this reaction was *cis*-hydroxylated at the double bond to give methyl 3 α -acetoxy-6 β ,7 β -dihydroxy-5 β -cholanoate by using OsO₄ in the presence of pyridine and reducing to the glycol with aqueous methanolic Na₂S₂O₄ (Kagan & Jacques, 1960). Hydrolysis of the product gave β -muricholic acid, m.p. 224–226° (uncorrected, with a Fisher–Jones apparatus). Kagan & Jacques (1960) reported m.p. 226°. A methylated sample of the acid run on a thin-layer plate with methanol–acetone–chloroform (1:5:14, by vol.) had the same *R_F* (0.22) as that of the methyl ester of a reference sample of β -muricholic acid run on an adjacent lane.

RESULTS

Two experiments were carried out, each with mitochondria and soluble supernatant fraction prepared from the pooled livers from a separate group of four rats. The two experiments gave qualitatively similar results, though there were minor differences in the relative amounts of the various radioactive compounds identified in the butanol extracts of the incubation mixtures. The results described below are from the first experiment.

Six flasks, each with 1 μ C of [4-¹⁴C]cholesterol in the incubation mixture containing rat-liver mitochondria and the soluble supernatant fraction, were incubated for 6 hr. at 37°. At the end of the incubation, the radioactive compounds were extracted into butanol. The washed butanol extracts from the six flasks were pooled, evaporated to dryness and submitted to thin-layer chromatography in chloroform–methanol (9:1, v/v). Radioautographs prepared from the thin-layer chromatograms showed five main bands of radioactivity (Fig. 1). Band I contained 7% of the total activity in the

butanol extract, band II contained 1.6%, band III contained 1.5% and band IV, which remained on the origin, contained about 4%.

Chromatography of band I. The radioactive material in band I ran with the *R_F* of cholesteryl benzoate. After alkaline hydrolysis of the eluate from this band, all the radioactivity was identified as free cholesterol.

Chromatography of band II. Band II was eluted with chloroform–methanol (2:1, v/v) and chromatographed in benzene–ethyl acetate (2:3, v/v) and in chloroform–methanol (9:1, v/v), with markers of 7 α -hydroxycholesterol, 26-hydroxycholesterol, 7-oxocholesterol and cholesterol. In both solvents, most of the radioactivity on the chromatogram coincided with the 26-hydroxycholesterol marker, but traces were also present in the positions of cholesterol and 7 α -hydroxycholesterol. Fig. 2 shows the results obtained with benzene–ethyl acetate.

Identification of the radioactive compound in band III. Band III was eluted with chloroform–methanol (1:1, v/v) and chromatographed in chloroform–methanol (9:1, v/v), in chloroform–methanol (3:1, v/v) and in benzene–ethyl acetate (1:2, v/v). In each solvent, the area of radioactivity ran as 3 β -hydroxycholestenic acid. Further evidence for the formation of this acid was obtained when non-radioactive 3 β -hydroxycholestenic acid was added at the beginning of the incubation. In

Fig. 1. Radioautograph of a thin-layer chromatogram of the butanol extract from an incubation with [4-¹⁴C]-cholesterol. The solvent was chloroform–methanol (9:1, v/v). Experimental details are given in the Methods section.

* The following trivial names are used: 7 α -hydroxycholesterol, cholest-5-ene-3 β ,7 α -diol; 7-oxocholesterol, 3 β -hydroxycholesterol-5-en-7-one; 26-hydroxycholesterol, cholest-5-ene-3 β ,26-diol; 3 β -hydroxycholestenic acid, 3 β -hydroxycholesterol-5-en-26-oic acid; 3 α ,7 α ,12 α -trihydroxycholestan-26-oic acid. The following acids are derivatives of 5 β -cholanoic acid, with the hydroxyl groups as shown: lithocholic acid, 3 α -; chenodeoxycholic acid, 3 α ,7 α -; deoxycholic acid, 3 α ,12 α -; cholic acid, 3 α ,7 α ,12 α -; hyocholic acid, 3 α ,6 α ,7 α -; α -muricholic acid, 3 α ,6 β ,7 α -; β -muricholic acid, 3 α ,6 β ,7 β -; ω -muricholic acid, 3 α ,6 α ,7 β -.

Fig. 2. Radioautograph of a thin-layer chromatogram of the eluate from band II (Fig. 1). Outlines of markers are shown by dotted lines. The solvent was benzene-ethyl acetate (2:3, v/v).

Fig. 3. Radioautograph of a thin-layer chromatogram of the methyl ester of an eluate from band III (Fig. 1) run on plates treated with AgNO_3 . The outline of a marker of the methyl ester of 3β -hydroxycholestenic acid is shown by the dotted line. The solvent was chloroform-acetone (24:1, v/v).

Table 1. *Effect of 3β -hydroxycholestenic acid on the accumulation of radioactive steroids formed from $[^{14}\text{C}]$ cholesterol incubated with rat-liver mitochondria*

3β -Hydroxycholestenic acid (1.5 mg./flask) was added to the incubation mixture as a suspension in 1% bovine serum albumin in tris-HCl buffer, pH 8.5 (0.1 M). The details of the incubation mixture are given in the Methods section. The butanol eluate from the incubation was chromatographed on thin-layer chromatography plates with chloroform-methanol (9:1, v/v) (see Fig. 1), and the radioactive bands were eluted and assayed for activity.

Radioactive band on thin-layer chromatography plate	Percentage of total activity eluted from plate	
	Control	Plus 3β -hydroxy- cholestenic acid
I	4.6	10.6
Cholesterol	88.1	81.0
II	1.4	4.6
III	1.4	2.8
IV	4.5	1.0

this experiment there was an increase in the proportions of the total activity in the butanol extract present in bands I, II and III, and a decrease in the proportion present in band IV (Table 1). A portion of the radioactive material from band III was eluted and methylated. The product was chromato-

graphed on thin-layer chromatography plates impregnated with silver nitrate (Avigan, Goodman & Steinberg, 1963), with chloroform-acetone (24:1, v/v) as solvent and the methyl ester of 3β -hydroxycholestenic acid as marker. A radioautograph of the chromatogram showed a main band of radioactivity corresponding to the marker (Fig. 3) and a faint band at the origin corresponding to free 3β -hydroxycholestenic acid.

Presence of conjugated bile acids in band IV. Band IV was eluted with ethanol and a portion of the eluate was chromatographed in system no. 2 of Hofmann (1962). The chromatogram showed four radioactive bands near the origin with R_f values similar to those reported by Hofmann (1964) for taurine conjugates of monohydroxy-, dihydroxy- and trihydroxy-cholanoic acid (Fig. 4). When the ethanol eluate from band IV was submitted to mild hydrolysis in *n*-sodium hydroxide in aq. 80% (v/v) dioxan at 90° for 2 hr., and was then acidified and extracted with diethyl ether, 70–80% of the total radioactivity remained in the aqueous phase. After hydrolysis of the ethanol extract under the conditions required for hydrolysis of conjugated bile acids (Mosbach *et al.* 1954), about 90% of the radioactivity in the acidified hydrolysate was extractable with

diethyl ether. When the ether extract was chromatographed in system no. 1 of Hofmann (1962), a radioautograph of the chromatogram showed three main bands of radioactivity, band *a* with R_F 0.62, band *b* with R_F 0.34 and band *c* with R_F 0.11. There was also a trace of radioactivity at the origin.

C₂₄ bile acids in band IV. Band *a*, containing about 27% of the activity in the ether extract, had the same R_F as lithocholic acid and 3 β -hydroxychol-5-enoic acid (which are not separated in Hofmann's system no. 1). Band *a* was eluted with ethanol and a portion of the dried eluate was crystallized five times from hot methanol with 100mg. of 3 β -hydroxychol-5-enoic acid. After the first crystallization, the specific activity fell to 21% of the calculated value and then remained constant after further recrystallization. Similar results were obtained when benzene was used for recrystallization. A second portion of the dried eluate of band *a* was crystallized five times from ethyl acetate with 100mg. of lithocholic acid. After the first crystallization, the specific activity fell to 78% of the calculated value and then remained constant. When the eluate from band *a* was methylated and chromatographed in chloroform-acetone (24:1, v/v) on thin-layer chromatography plates impregnated with silver nitrate (Avigan *et al.* 1963), the chromatogram showed two radioactive bands, one corresponding to the methyl ester of lithocholic acid and the other to the methyl ester of 3 β -hydroxychol-5-enoic acid (Fig. 5).

Band *b*, containing about 38% of the activity in

the ether extract, had the same R_F as deoxycholic acid and chenodeoxycholic acid in system no. 1 of Hofmann (1962). A portion of the eluate from band *b* was crystallized four times from ethyl acetate-hexane with 200mg. of chenodeoxycholic acid. After the first crystallization the specific activity fell to 48% of the calculated value and then remained constant after further recrystallization. Another portion of the eluate from band *b* was crystallized six times from acetone-water with 200mg. of 3 α ,6 β -dihydroxy-5 β -cholanoic acid. After the second crystallization the specific activity fell to 42% of the calculated value and then remained constant after four further recrystallizations. Thin-layer chromatography of the eluate from band *b* with acetic acid-di-isopropyl ether-iso-octane (1:1:2, by vol.) showed two bands of radioactivity (Fig. 6), one coinciding with chenodeoxycholic acid and the other with 3 α ,6 β -dihydroxy-5 β -cholanoic acid. No activity was detectable at positions corresponding to the R_F values of deoxycholic acid and 3 α ,6 α -dihydroxy-5 β -cholanoic acid, both of which were well separated

Fig. 4. Radioautograph of a thin-layer chromatogram of the eluate from band IV (Fig. 1). Mobilities of free bile acids and of glycine and taurine conjugates of bile acids are shown by brackets (calculated from R_F values given by Hofmann, 1964). The solvent was system no. 2 of Hofmann (1962).

Fig. 5. Radioautograph of a thin-layer chromatogram of the methyl esters of an eluate from band IVa. Outlines of markers of the methyl esters of lithocholic acid and 3 β -hydroxychol-5-enoic acid are shown by dotted lines. The solvent used was chloroform-acetone (24:1, v/v), with an AgNO₃-treated plate.

from chenodeoxycholic acid and $3\alpha,6\beta$ -dihydroxy- 5β -cholanoic acid (Fig. 6).

Band c, containing about 35% of the activity in the ether extract from the hydrolysate, moved slightly faster than cholic acid in system no. 1 of Hofmann (1962). When the eluate from this band was methylated and chromatographed in methanol-acetone-chloroform (1:5:14, by vol.), two main bands of radioactivity were obtained. The more intense of these corresponded to the methyl ester of α -muricholic acid, and the other to the methyl ester of β -muricholic acid (Fig. 7). There was a faint trace of blackening behind the methyl α -muricholate band at a position corresponding to methyl cholate. No activity was detectable at the positions corresponding to the methyl esters of hyocholic acid and ω -muricholic acid. A portion of the eluate from band c was crystallized seven times from methanol-water with 90 mg. of β -muricholate. After the third crystallization, the specific activity fell to 24% of the calculated value and then remained constant.

DISCUSSION

Cholic acid is the predominant bile acid excreted in the bile of rats. According to the current view on

how cholesterol is converted into cholic acid *in vivo*, saturation of the Δ^5 -double bond, α -hydroxylation at positions 7 and 12 and epimerization of the hydroxyl group at C-3 take place before oxidation and cleavage of the side chain (see Danielsson, 1963). The main evidence for this is the finding that cholic acid is formed *in vivo* from 7α -hydroxycholesterol (Bergström & Lindstedt, 1956) and from $3\alpha,7\alpha,12\alpha$ -trihydroxy- 5β -cholestane (Bergström, Pääbo & Rumpf, 1954), in both of which compounds the side chain is intact, but is not formed from 26-hydroxycholesterol (Danielsson, 1961) or from 3β -hydroxycholesterol-5-enoic acid (Bergström, 1955).

Isolated liver mitochondria catalyse the oxidative cleavage of the side chain of cholesterol, and convert $3\alpha,7\alpha,12\alpha$ -trihydroxycholestanoic acid into cholic acid (Briggs, Whitehouse & Staple, 1961). However, attempts to show that they can also convert cholesterol into cholic acid *in vitro* have not been successful. This raises the question whether observations made on cell-free systems *in vitro* give valid information about the formation of bile acids in the whole animal. Our observations confirm those of other workers in showing that isolated liver mitochondria form little or no cholic acid from cholesterol, presumably because they are deficient in the enzyme system that catalyses α -hydroxylation at position 12. They show, however, that liver mitochondria in the presence of the soluble supernatant fraction catalyse the introduction of α -hydroxyl groups at positions 3 and 7, and of β -hydroxyl groups at positions 6 and 7, with the formation of lithocholic acid, chenodeoxycholic acid and α - and β -muricholic acids. All of these, including α - and β -muricholic acids (Hsia *et al.* 1957, 1958), are normally present in rat bile.

Fig. 6. Radioautograph (left) of a thin-layer chromatogram from the eluate from band IVb, with positions of markers of chenodeoxycholic acid and $3\alpha,6\beta$ -dihydroxy- 5β -cholanoic acid shown by dotted lines; markers of four dihydroxy acids were run on an adjacent lane on the same thin-layer plate (right). The solvent used was acetic acid-di-isopropyl ether-iso-octane (1:1:2, by vol.).

Fig. 7. Radioautograph (lower) of a thin-layer chromatogram of the methylated eluate from band IVc, with the positions of the markers of the methyl esters of α - and β -muricholic acids shown by dotted lines; a diagram of positions of markers of the methyl esters of five trihydroxycholanoic acids run on the same thin-layer plate (upper) is shown. The solvent used was methanol-acetone-chloroform (1:5:14, by vol.). O, Origin.

Almost all the C₂₄ bile acids recovered in the butanol extract, including 3 β -hydroxychol-5-enoic acid, were in the form of conjugates that released free bile acid after hydrolysis under the conditions required to break peptide bonds, but not after hydrolysis under milder conditions. This, together with the behaviour of the polar fraction IV (Fig. 1) on thin-layer chromatography in Hofmann's system no. 2 (Fig. 4), suggests that the C₂₄ bile acids formed in the incubation mixture were present as taurine conjugates. Bremer & Gloor (1955) and Siperstein & Murray (1956), who have demonstrated the formation of taurocholic acid from taurine and cholic acid in cell-free preparations of liver, concluded that the enzymes necessary for this synthesis are present mainly in the microsomes and the cell sap. Our findings do not necessarily conflict with this conclusion, since the concentrations of bile acids present in our incubation mixture must have been much less than the concentration of cholic acid added in the experiments of Bremer & Gloor (1955) and of Siperstein & Murray (1956). The present work therefore suggests that mitochondria in the presence of the soluble supernatant fraction can synthesize conjugated bile acids efficiently when the concentration of substrate is low, but not when it is high. Our failure to find any 3 β -hydroxycholestenic acid in the hydrolysate from band IV shows that this acid is not converted into a conjugated form under our conditions.

Chenodeoxycholic acid is probably formed *in vivo* mainly by a pathway analogous to that for cholic acid, with 7 α -hydroxycholesterol and 3 α ,7 α -dihydroxy-5 β -cholestane as early intermediates (Bergström, Danielsson & Samuelsson, 1960). However, there is evidence for another pathway in which the first step is the oxidation of the side chain of cholesterol. Fredrickson & Ono (1956) have shown that 26-hydroxycholesterol is formed when cholesterol is incubated with liver mitochondria. Danielsson (1961) has confirmed this and has shown that 26-hydroxycholesterol, when injected into rats, gives rise to chenodeoxycholic acid and to two trihydroxylated acids similar to α - and β -muricholic acids. In the light of these and other observations, Danielsson (1963) has suggested a pathway for the synthesis of chenodeoxycholic acid from cholesterol *in vivo*, in which the first step is the formation of 26-hydroxycholesterol, followed by 7 α -hydroxylation to give cholest-5-ene-3 β ,7 α ,26-triol, which is then converted into chenodeoxycholic acid. The results of our experiments *in vitro* are consistent with Danielsson's (1963) suggestion that 26-hydroxycholesterol is the first intermediate in one of the pathways by which chenodeoxycholic acid is formed from cholesterol. However, the presence of radioactive 3 β -hydroxycholestenic acid and 3 β -hydroxychol-5-enoic acid in the incubation mixture

shows that the oxidative removal of the isopropyl unit of the cholesterol side chain can be completed before any modification of the ring system occurs, and suggests the scheme shown in Scheme 1 for the formation of chenodeoxycholic and other C₂₄ acids by liver mitochondria *in vitro*. The sequence of changes between lithocholic acid and α - and β -muricholic acids is based on the observations by Thomas *et al.* (1964, 1965) on the metabolism of lithocholic acid and 3 α ,6 β -dihydroxy-5 β -cholanoic acid in intact rats. Evidence for the conversion of 3 β -hydroxychol-5-enoic acid into chenodeoxycholic acid via lithocholic acid has been given elsewhere (Mitropoulos & Myant, 1966b). The assumption that the radioactive 3 β -hydroxycholestenic acid isolated from the incubation mixture is converted into C₂₄ bile acids is supported by our finding that the addition of non-radioactive 3 β -hydroxycholestenic acid diminishes the yield of radioactive C₂₄ acids from [¹⁴C]cholesterol.

If the pathway shown in Scheme 1 occurs in the intact rat, some of the lithocholic acid present in bile may be synthesized in the liver. This is contrary to the generally accepted view that all the lithocholic acid excreted in the bile is formed in the large intestine through removal of the 7 α -hydroxyl group of chenodeoxycholic acid by bacterial action. However, the suggestion that lithocholic acid may to some extent be a primary bile acid is in keeping with the observation by Siperstein, Harold, Chaikoff & Dauben (1954) that radioactive lithocholic acid is excreted in the bile of bile-fistula rats injected with [4-¹⁴C]cholesterol.

Our observations show that the liver brings about the conversion of cholesterol into 3 β -hydroxychol-5-enoic acid, and other workers have shown that cholic acid can be formed from 3 α ,7 α ,12 α -trihydroxycoprostanol (Bergström *et al.* 1954). The liver therefore contains enzymes capable of catalysing all the steps in the oxidation and cleavage of the cholesterol side chain, either before saturation of the Δ^5 -double bond and α -hydroxylation at positions 3, 7 and 12, or after modification of the ring system has been completed. Further, the liver enzymes catalysing saturation of the ring system and α -hydroxylation at positions 3 and 7 can use as substrates compounds with an intact cholesterol side chain or with a side chain from which the isopropyl unit has been removed. Thus 3 α ,7 α -dihydroxy-5 β -cholestane can be formed from cholesterol (Mendelsohn, Mendelsohn & Staple, 1965), and chenodeoxycholic acid can be formed from cholanoic acid (Ray *et al.* 1961) and, as shown in the present work, from 3 β -hydroxychol-5-enoic acid.

The apparent lack of specificity of the liver enzymes concerned in the oxidative cleavage of the side chain and in the modification of the ring

Scheme 1. Suggested scheme for the formation of C₂₄ bile acids from cholesterol by liver mitochondria *in vitro*. I, Cholesterol; II, 26-hydroxycholesterol; III, 3 β -hydroxycholestenic acid; IV, 3 β -hydroxychole-5-enoic acid; V, lithocholic acid; VI, chenodeoxycholic acid; VII, 3 $\alpha,6\beta$ -dihydroxy-5 β -cholanoic acid; VIII, α -muricholic acid; IX, β -muricholic acid.

system of cholesterol may explain why several pathways appear to be followed in the formation of chenodeoxycholic acid. If the modifications to the ring system can occur at any stage during the oxidative cleavage of the side chain, then the pathway followed by a given molecule of cholesterol in an intact liver cell may depend on the extent to which its side chain has been oxidized before it reaches the enzymes catalysing ring saturation and α -hydroxylation at positions 3 and 7. On this view, several pathways from cholesterol to chenodeoxycholic acid are possible in the intact cell, though some are likely to be more important than others. These considerations do not apply to cholic acid, since the pathway from cholesterol to cholic acid in rats is restricted by the fact that α -hydroxylation at position 12 cannot occur once the first step in the oxidation of the side chain has taken place.

It is impossible to say to what extent the pathway shown in Scheme 1 takes place *in vivo*. However, the formation of several naturally occurring bile acids in the system *in vitro* shows that mitochondria in the presence of the soluble supernatant fraction can carry out the oxidative cleavage of the side chain, the saturation of the Δ^5 -double bond and all but one of the hydroxylations in the ring system. To this extent, investigation of the metabolism of cholesterol by liver mitochondria in the system we have used seems to be relevant to the problem of the synthesis of bile acids in the whole animal.

The following kindly gave us reference compounds: Dr S. L. Hsia (3 α ,6 β -dihydroxy-5 β -cholanoic acid, α - and β -muricholic acids), Dr V. Černý (26-hydroxycholesterol and 3 β -hydroxycholestenic acid), Dr O. Wintersteiner (7 α -hydroxycholesterol) and Dr P. D. G. Dean (ω -muricholic acid).

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